

Comparative Virologic Studies of Condylomata Acuminata Reveal a Lack of Dual Infections with Human Papillomaviruses

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Condylomata acuminata are epithelial proliferations caused by infection of the anogenital squamous epithelium with human papillomavirus (HPV). DNA-DNA hybridization techniques and the extremely sensitive polymerase chain reaction (PCR) were used to analyze biopsies from patients with clinically diagnosed condyloma acuminatum for the presence of HPV DNA. PCR analyses using primers and oligonucleotide probes specific for the E6/E7 region of HPV-6, -11, or -16 showed that 31 (93.9%) of 33 tissue biopsies contained HPV DNA: 22 contained type 6 DNA, 6 contained type 11 DNA, and 3 contained type 16 DNA. Eleven biopsies positive by PCR were Southern hybridization-negative or were considered inadequate for Southern analysis. In all 11, the presence of HPV DNA was corroborated by the observation of histopathologic evidence suggestive of HPV infection or by *in situ* hybridization. No evidence of multiple infections with HPV-6 or -11 and HPV-16 was seen.

Condyloma acuminatum is the most common viral sexually transmitted disease in the United States [1]. These genital warts are caused by human papillomavirus (HPV) infection of the anogenital squamous epithelium. About 60 distinct HPV genotypes have been recognized, of which some 20 infect the anogenital mucosa and skin [2]. Often, but not always, specific HPVs are associated with distinct clinical and histopathologic characteristics, such as HPV-6 or -11 with condylomata and HPV-16 with squamous-cell precancers and carcinomas. These associations are not invariable, as HPV-6 has been found in anogenital squamous cell carcinomas and HPV-16 in some cutaneous condylomata (for review see [3]). Also, although condylomata are generally benign, several studies have suggested that the presence or a history of condylomata is related to the development of squamous-cell carcinoma [3]. To further characterize the association of HPV infection with benign anogenital neoplasia, we used polymerase chain reaction (PCR) amplification [4] to compare virologic and histopathologic characteristics of condylomata acuminata.

Materials and Methods

Condyloma specimens. Anogenital condyloma tissues were collected from 35 patients attending three Seattle-area community-based clinics. Condyloma acuminatum was diagnosed by gross appearance of the lesion and clinical history of the patient. The tissue available

for laboratory analyses was usually a shave or punch biopsy; for 18, part of the lesion was fixed in formalin and embedded in paraffin.

Southern filter hybridizations. Total cellular DNA was prepared from each specimen. Depending on the yield of cellular DNA, 1-5 μ g of DNA was digested with *Bam*HI plus *Eco*RI and with *Pst*I (Bethesda Research Laboratories, Gaithersburg, MD). Restriction products were electrophoresed through agarose gels and transferred to nitrocellulose by Southern's method [5]. Filters were hybridized in stringent conditions as described [6] with ³²P-labeled HPV-6, -11, or -16 DNA; separately and sequentially hybridized with HPV-16, -6, and -11 probes; and exposed for 7 days.

PCR. The sequences for oligonucleotide primers and probes were derived from nonconserved regions of the E6/E7 open-reading frames of HPV-6, -11, and -16. These sequences for HPV-6 and -16 have been described elsewhere [7]. For HPV-11, the primer sequences were TGTGTGGCGAGACAACCTTTCCTTT (nucleotides 260-284) and AAGCGTGCCTTTCCCAATATGTGCT (nucleotides 478-464), and the oligonucleotide probe sequence was ATAGACACTTTAAT-TATGCTGCATATGCACCTACAGTAGA (nucleotides 331-370). Purified DNA (250 ng) from condyloma tissues was amplified as described [7]. Plasmid HPV-6, -11, and -16 DNAs were used as controls. A set of consensus amplification primers (derived from conserved sequences in the L1 region of HPV-6, -11, -16, -18, and -33) [8] were used to confirm some results. PCR products were digested with *Rsa*I (Amersham, Arlington Heights, IL) to allow distinction between genital HPV types [8].

PCR products were transferred to nitrocellulose and hybridized with type-specific (for 6, 11, and 16) or L1 consensus oligomeric probes. After autoradiography, amplification products ≥ 0.01 fg of HPV DNA (~ 10 molecules) could be detected. The sequence of our consensus probe, different from that of Ting and Manos [8], was CATGTGGAGGARTATGATTACAGTTTATT (nucleotides 6869-6898 in HPV 6; R = A or G). It was designed to hybridize with different-sized *Rsa*I digestion fragments (see figure 1). Meticulous care was taken in handling DNA samples and reagents to prevent crossover contamination [9].

***In situ* hybridization and histopathology.** Formalin-fixed, paraffin-embedded tissue sections (18) were hybridized with biotinylated

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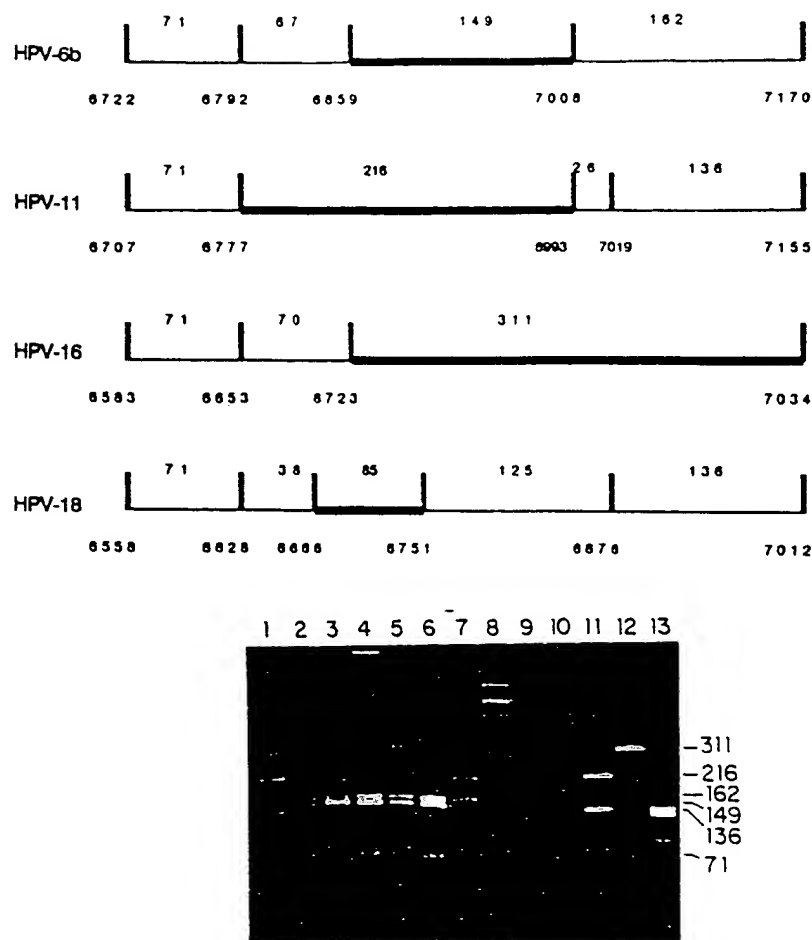


Figure 1. Polymerase chain reaction analysis of condyloma DNA with consensus primers followed by *RsaI* digestion. Line drawing shows *RsaI* restriction fragments of HPV-6, -11, -16, and -18 late region amplimers. Heavy lines show fragments that hybridize with consensus probe (sequence indicated by vertical dashed lines). Ethidium bromide-stained gel shows *RsaI* digestion products of amplimers generated from these sequences: lanes 1 and 4, vaginal lesions; 2 and 7, penile lesions; 3, perineal lesion; 5, perianal condyloma; 6, penile verrucous lesion; 8, *AluI*-digested pBR322 DNA; 9, negative control; 10–13, plasmid HPV-6, -11, -16, and -18 DNA, respectively (unless indicated, histology of lesion is unknown). Amounts used were 250 ng of original specimen DNA and 1 pg of plasmid DNA. HPV DNA in lane 7 is missing *RsaI* site at nucleotide 6859, resulting in three fragments of 216, 162, and 71 bp.

HPV-6, -11, or -16 DNA probes in stringent conditions [10]. Hybridized DNA was detected by sequential immunocytochemical reactions and visualized with a light microscope as described [10].

Two-stopped hematoxylin and eosin-stained sections from the tissues examined by in situ hybridization were reviewed by one pathologist (D.M.) for histopathologic changes indicative of HPV infection. For one other subject, a slide but not a block was available. For each, the presence of papillomatosis, acanthosis, hyperkeratosis, hypergranulosis, parakeratosis, and koilocytosis was noted.

Results

Southern hybridization. Of 35 biopsies obtained, 28 were examined by Southern hybridization (table 1). Seven were considered inadequate for Southern analyses as <1 µg of cellular DNA was purified. Of the 28, 20 (71%) were positive for HPV DNA: 7 (35%) for HPV-6, 2 (10%) for HPV-11, 2 (10%) for HPV-16, and 9 (45%) for either HPV-6 or -11 (indistinguishable by restriction endonuclease digestion and hybridization analyses). For the latter 9, *Bam*HI plus *Eco*RI digestions gave linear viral DNAs of ~7.9 kbp, the expected digestion pattern for both HPV-6 subtype a and HPV-11 because each of these genomes has no *Eco*RI site [11]. DNA was insufficient for *Pst*I digestion.

Table 1. Detection of human papillomavirus (HPV) DNA in anogenital condylomata by hybridization and polymerase chain reaction (PCR).

| | Histopathology | | | |
|------------------------|--------------------|---|----------------------|---------------------|
| | Typical (n = 8) | Without koilo- cytosis (n = 5) | Verrucous (n = 6) | Unknown (n = 16) |
| Negative | 1 | 0 | 0 | 1 |
| Positive by | | | | |
| Southern hybridization | 5* | 0† | 2‡ | 13 |
| In situ hybridization | 7 | 0§ | 0 | NA |
| PCR | 7 | 5 | 6 | 13 |
| HPV type | | | | |
| 6 | 5 | 4 | 5 | 8 |
| 11 | 2 | 0 | 1 | 3 |
| 16 | 0 | 1 | 0 | 2 |

NOTE. Subjects were 23 women (ages, <18 to 47 years; median, 29) with lesions on vulva (10), vagina (8), perineum (2), perianal area (1), and unspecified anogenital area (2); 12 men (ages, <18 to 55 years; median, 27) with lesions on penis (10), perianal area (1), and groin (1). PCR used type-specific E6/E7 primers and oligonucleotide probes. NA = not available.

* One specimen was typed by PCR on formalin-fixed tissue; no Southern analysis was done.

† Two specimens were inadequate for Southern analysis.

‡ Four specimens were inadequate for Southern analysis.

§ In situ hybridization could not be done on one specimen.

|| Two specimens were inadequate for PCR.

PCR. DNA from 32 tissues was examined by PCR with primers specific for the E6/E7 region of HPV-6, -11, and -16 (table 1). One sample was received by surface mail, and its DNA was too degraded for either Southern hybridization or PCR analysis. PCR was done on one paraffin-embedded section [12] from this condyloma. Two biopsies were not tested by PCR as all of the DNA was used in Southern hybridizations.

By PCR, 31 (93.9%) of 33 tissues contained HPV DNA: 22 (71%) of the 31 contained HPV-6 DNA, 6 (19%) HPV-11, and 3 (10%) HPV-16. No evidence of multiple infections was seen. No sex- or site-specific associations were noted for lesions containing HPV-6 or -11 DNA. Of the 3 that contained HPV-16 DNA, 2 were located on the vulva and 1 on the penis of the affected individuals.

PCR using consensus primers and probes was done on 19 of the 35 samples (because of the limited material available from shave or punch biopsies). After amplification with the consensus primers, DNAs were restricted with *RsaI* before being electrophoresed and hybridized. The map of *RsaI* digestion products for HPV-6, -11, -16, and -18 is shown in figure 1. *RsaI* digestion of consensus amplimers can also be used to distinguish HPV-31, -33, and -35 (data not shown).

PCR results obtained with the consensus primers and probes agreed with those from the E6/E7 primers for 16 of the 19 condyloma DNAs. Two that were HPV-6-positive with the E6/E7 primers gave ambiguous results with the consensus primers. One positive with the E6/E7 primers was negative with the consensus primers. All five that were HPV-11 and the one HPV-16 with the E6/7 primers were the same type with the consensus primers.

Histopathology and in situ hybridization. A total of 26 tissue blocks from 19 patients were reviewed for histopathologic evidence of condyloma acuminatum (table 1). Three distinct patterns were observed: a typical condylomatous change with papillomatosis, acanthosis, diffuse parakeratosis, koilocytosis, and with or without hyperkeratosis; acanthosis, some papillomatosis, and slight parakeratosis suggestive of condyloma but without obvious koilocytotic cells, showing more cellular atypia than typical condylomata with numerous mitoses and basaloid cells in the epithelium; and warty without condylomatous features appearing as excrescences, with acanthosis, variable hyperkeratosis and parakeratosis, and no koilocytosis.

Eight of the 19 lesions were considered typical condylomata acuminata, and seven contained either HPV-6 or -11 DNA (table 1). One was negative for HPV-6, -11, and -16 DNA by Southern and in situ hybridization and by PCR. Cellular DNA from this tissue was inadequate for analysis with the late consensus primers. Presumably this lesion contained another HPV type associated with condyloma acuminatum. Five lesions were designated condylomata without obvious koilocytosis, and six lesions were verrucous.

All samples except the one for which only a slide was available were examined by in situ hybridization with biotinylated

HPV-6, -11, and -16 DNA probes (table 1). For the seven condylomata that were positive by in situ hybridization and typed by PCR, the results were concordant.

Comparative virologic studies. Twenty specimens were positive by both Southern hybridization and PCR (table 1). For DNAs definitively typed by Southern hybridization, the PCR results were in perfect agreement (seven HPV-6, two HPV-11, and two HPV-16). For nine considered either -6 or -11 by Southern hybridization, PCR showed that five were HPV-6 and four were HPV-11.

Four specimens were negative by Southern hybridization but positive for HPV-6 DNA by PCR. All four had histopathologic evidence of condylomata, one typical and three without obvious koilocytosis. The typical lesion also was HPV-6 DNA-positive by in situ hybridization.

Seven specimens were considered inadequate for Southern hybridization analyses but were positive for HPV DNA by PCR. From six of these, <1 μ g of cellular DNA was recovered, and the DNA from one was completely degraded because it had been sent by surface mail. By PCR, six specimens contained HPV-6 and one HPV-16 DNA. One of these was a typical condyloma, and the others had histopathologic evidence suggestive of HPV infection (four verrucous and two condylomata without obvious koilocytosis).

Two DNA specimens were negative by both Southern hybridization and PCR. One of these, with only a single section available, was considered a typical condyloma by histopathology. Neither specimen had adequate cellular DNA for analysis with consensus primers. Two specimens were Southern hybridization-negative and inadequate for PCR analysis, and no tissue was available for further analysis.

Discussion

In this study, 31 (93.9%) of 33 tissues analyzed by PCR contained detectable HPV-6, -11, or -16 DNA sequences from the E6/E7 region of the viral genomes, and in 20 of these Southern hybridization results were in complete agreement as to the HPV genotype. Eleven lesions contained HPV DNA by PCR, but 4 had no detectable viral DNA by Southern hybridization and 7 had inadequate cellular DNA for Southern analysis. For each of these, the positive PCR result was corroborated by a positive in situ hybridization result or by histopathologic evidence suggestive of HPV infection.

This study is a small part of a large population-based case-control study of risk factors associated with the development of condylomata acuminata. In this group, HPV-6 and -11 were the most commonly associated with anogenital cutaneous condylomata, although HPV-16 DNA was found in 10% of HPV-positive lesions. No evidence of infection with more than one HPV type was seen. In other studies, HPV-6 or -11 were also the most common [13-15]. However, these studies found somewhat differing prevalences of HPV-16 occurring alone and varying proportions of condylomata that contained more than

one type of HPV. Estimates of the proportion of condylomata positive for HPV-16 alone range from none [13, 14] to ~10% (this study) [15]. Rates of multiple infections (with HPV-6, and -16 or -6 and -18) range from none or <1% (this study) [13, 14] to 13% [15]. As these investigations used different populations and methods (in situ hybridization with either biotinylated DNA probes [14] or radioactive RNA probes [13] and Southern hybridization [15]), generalizations among them cannot be made.

An important finding was that 11 of 19 lesions clinically identified as condylomata were suggestive of but not unequivocally typical condylomata by histologic criteria. By PCR analysis or Southern hybridization, all 11 contained HPV DNA of types commonly found in genital warts. These results agree with those of Wilbur et al. [13], who found HPV mRNA in genital lesions they considered "suggestive but not diagnostic for HPV infection." Nuovo et al. [14] also found HPV DNA by Southern but not by in situ hybridization in histologically "nondiagnostic" lesions, as was the case in our study. "Suggestive" and "nondiagnostic" are analogous to our "condylomata without obvious koilocytosis" and "verrucous changes" in that lesions lacked unequivocal koilocytotic changes in keratinocytes but showed other changes such as acanthosis, parakeratosis, or hyperkeratosis. These results demonstrate that a significant proportion of exophytic anogenital squamous lesions that do not meet the classic diagnostic criteria for condyloma are, in fact, associated with HPV infection.

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